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14. ABSTRACT
In this research project, we investigated the hypothesis that mutation of the NF1 gene might alter axonal and dendritic transport in neurons. This might provide new insights into the development of cognitive defects in patients with neurofibromatosis 1. The significant results of this pilot research project are all derived from functional studies done by sciatic nerve ligation in wild-type and Nf1-/+ mice. After nerve ligation, proximal and distal segments of the sciatic nerve were homogenized, and analyzed for content of tubulin and synaptic proteins by Western blot and immunohistochemistry. Out results support the idea that there is a quantitative decrease in the accumulation of synaptic proteins immediately proximal to the site of nerve ligature in the Nf-/+ nerve, as compared to wild-type nerves. This supports the idea that Nf1 gene mutation does indeed result in a quantitative defect in axonal transport in the peripheral nervous system. We are preparing to see if there is a similar deficiency in the rate of axonal transport in the central nervous system (CNS) as well. Once we can confirm these findings in the CNS, then we shall embark on a series of experiments that address the molecular mechanisms by which axonal transport is affected in the Nf1-/+ mice.

15. SUBJECT TERMS
Axonal transport; synaptic proteins; sciatic nerve ligation; tubulin; synaptotagmin

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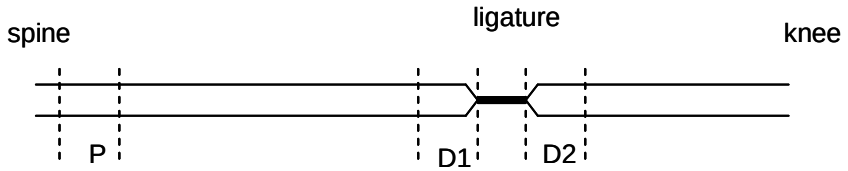
Table of Contents

	Page
Introduction……..	**1**
Body……..	**1-4**
Key Research Accomplishments……..	**4**
Reportable Outcomes……	**4**
Conclusion……	**4**
References……	**5**
Appendices……	**5-6**
Personnel ……..	**6**
Supporting Data ……...	**7**

Introduction:
The long term goal of our research is to understand the cellular and molecular mechanisms that lead to cognitive disturbance in children with neurofibromatosis 1. Previously published work [1, 2, 3] (from our and other labs) suggested an important interaction between neurofibromin (protein product of the Nf1 gene), kinesins, and amyloid precursor protein (APP). Based on this, we hypothesized that mutation of the Nf1 gene might affect axonal and dendritic transport, and this effect may contribute to neuronal dysfunction. The first aim of this project was an analysis of the expression of kinesins in wild-type and Nf1-/+ brain tissue (by in situ hybridization and immunohistochemistry). The second aim of this project was to directly compare axonal transport in wild-type and Nf1-/+ *in vivo* (in the sciatic nerve) and in vitro (in cultured neurons). Most of our results pertain to aim 2, the studies of axonal transport *in vivo*. We describe here the results of these studies.

Body:
As the primary aim of this project was to determine if there was a dysfunction of axonal transport in the Nf1-/+ mice, most of our efforts were directed towards specific aim 2.

Methods: The first several months of the period covered by this award were spent in the perfection of the surgical and analytical techniques required for studying axonal transport in the sciatic nerve. As described in the original Statement of Work, we initially performed sciatic nerve ligation experiments in P1 (postnatal age) mice (as described by Yonekawa et al [4]). We found that the sciatic nerves of these mice were too delicate to permit ligation with a surgical suture, and they also did not tolerate the prolonged anesthesia (3 hours). To get around this problem, we performed the experiments in 3-4 week old mice. A second major change that we made in our protocol after the first attempt at nerve ligation with surgical sutures, was to use aneurysm clips for nerve ligation. These aneurysm clips were provided to us by Dr. Mark Preul (Dept of Neurosurgery, Barrow Neurological Institute), and were easy to apply and provided a complete ligation of the nerve, without transecting the nerve. Mice were lightly sedated by inhalation of isoflurane, and then anesthetized by intraperitoneal injection of a ketamine/xylazine/acepromazine cocktail. Mice were kept under anesthesia during the entire experimental period (5 hours) and kept on a warming blanket. The sciatic nerves were exposed and the nerves were ligated using an aneurysm clip. The wound was covered and kept moist by irrigation with saline for the duration of the experiment. At the end of the experiment, mice were euthanized, and the entire nerve (from spine to below the knee) was removed and used for immunohistochemistry or for Western blot analysis. For Western blot analysis, 3 mm segments of each nerve were cut, and frozen separately. The segments taken were labeled P (proximal, at the spine), D1 (segment immediately proximal to the ligature), and D2 (immediately distal to the ligature), as shown in the figure below.



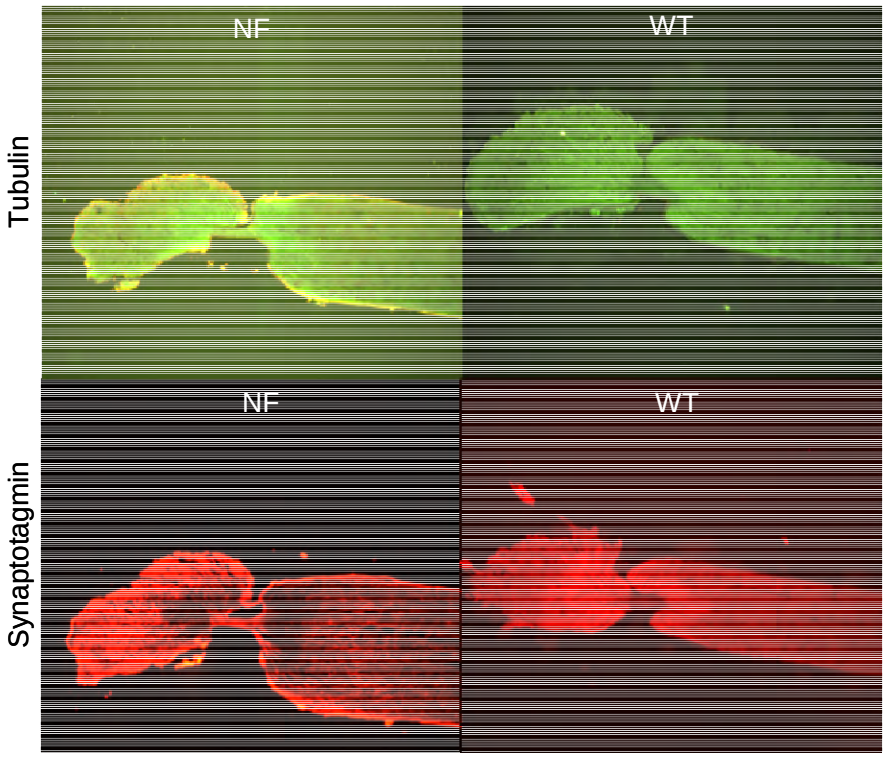
A sample of liver tissue was also taken from each mouse, for DNA extraction and genotyping. In this manner, the individual nerve segments are identified as either WT (wild-type) or NF (Nf1-/+). Protein homogenates were prepared from pools of 6 nerve segments of each genotype i.e. 6 proximal (P), 6 D1, and 6 D2 segments each from WT and NF animals. Nerve segments were homogenized in a nerve lysis buffer (0.1% SDS/0.5% deoxycholate/0.5% NP-40/10% Sigma

protease inhibitor cocktail/in PBS) and centrifuged to remove insoluble material. Protein concentration in each lysate was determined using the BCA protein assay kit (Pierce Biologicals, cat no. 23225). Lysates were then subjected to SDS-PAGE through 12% Novex Tris-glycine precise gels (Invitrogen), loading about 10 ug of protein in each well. After electrophoresis, electrophoretic transfer was done using nitrocellulose, and processed for Western analysis using the following primary antibodies: anti-tubulin (rabbit polyclonal antibody against neuron specific class III tubulin, Abcam ab18207; 1:1000 dilution), and anti-synaptotagmin (rabbit polyclonal antibody, Abcam ab10104; 1:500 dilution). Blots were blocked with 3% BSA in PBS. Primary antibody incubations were done overnight at 4^{o}C. The blots were then washed and incubated with secondary antibody (HRP-conjugated anti-rabbit IgG, Novus NB7160; dilution 1:30,000) at room temperature for 2 hours. Visualization of the Western blot was done by chemiluminescence using the Super Signal West Pico substrate (Pierce Biologicals, cat no. 34080), followed by exposure to X-ray film. These were scanned and imported into

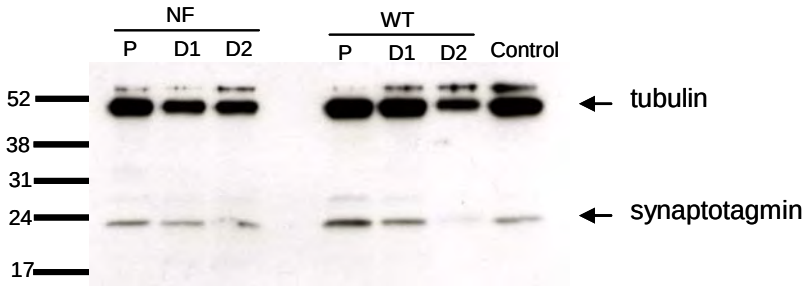
For immunohistochemistry, following nerve ligation as described above, the segment around the ligature (D1 + D2) was snap frozen, and embedded in OCT medium and frozen. Cryosections (10 um thick) were prepared and processed for immunohistochemistry. Briefly, sections were sixed in 4% bufferend paraformaldehyde, permeabilized with 0.2% Triton X100, blocked with blocking buffer (10% goat serum in PBS + 0.1% triton X-100). Sections were then incubated with anti-tubulin (R&D Systems, cat no. MAB1195) or anti-synaptotagmin (Sigma, cat no. S2177) overnight. Anti-tubulin was detected with Alexa Fluor 594-conjugated anti-mouse antibody, and anti-synaptotagmin was detected with Alexa Fluor 488-conjugated anti-rabbit antibody. Sections were photographed using an epifluorescence microscope.

Results:

Task 2: We initially performed immunohistochemical studies on ligated nerve segments from WT or NF mice, using antibodies against tubulin (a control antigen) and synaptotagmin and syntaxin -1 (synaptic proteins that are transported anterograde down the axon).



The figure above shows fairly uniform staining with anti-tubulin of the WT and NF nerves, both in the D1 and D2 segments. In contrast, there appears to be an accumulation of synaptotagmin in the D1 segment (immediately proximal to the ligature) of WT nerves, which is not as easily perceived in the NF nerve segment. These qualitative experiments lent support to our ideas, but also clearly pointed out the need for a quantitative assay method for differentiating between small changes in protein accumulation in the D1 segment.
For this purpose, we turned to Western blot analysis. We first determined that approximately 608 nerve segments (3 mm each) were required for extraction of a sufficient amount of protein to allow Western analysis. Secondly, because of the difference in molecular weight between tubulin (50kDa) and synaptotagmin (24 kDa), we were able to detect both antigens in a single blot at the same time (by incubating the blot with a mixture of the two primary antibodies). Shown in the figure below is the result of a Western blot of the WT and NF nerve segments.



The image was digitized and analyzed using the VisionWorksLS software (UVP, Inc.). The intensity of each synaptotagmin band was normalized by dividing by the intensity of the tubulin band in the same lane (S-T ratio). For each experiment we then calculated a numerical representation of the fractional accumulation of synaptotagmin in the D1 segment, comparing NF to WT (D1-NF/WT). We did this by computing the ratio of D1-NF (S-T ratio) / D1- WT (S-T ratio). Similarly, we also calculate a numerical measure of transport of synaptotagmin out of the post-ligature segment (D2).

D1-NF/WT = D1-NF(S/T ratio) / D1-WT(S/T ratio)

D2-NF/WT = D2-NF(S/T ratio) / D2-WT(S/T ratio)

We have done three replicates of this experiment, and find that the mean ratio of synaptotagmin accumulation in the D1 segment, D1-NF/WT, is 0.82 (+/- 0.05 SD). This suggests that following nerve ligation for 5 hours, there is a roughly 20% decrease in accumulation of synaptic proteins proximal to the ligature, implying that the rate of anterograde axonal transport is decreased in the NF nerves.

Conversely, we find that the mean ratio representing synaptotagmin exit from the D2 segment, D2-NF/WT, is 4.24 (+/- 3.17 SD). This suggests that following nerve ligation for 5 hours, synaptotagmin exits the D2 segment much slower in NF axons than in WT axons.

We expect to perform another three replicates of this experiment in the coming months (along with additional controls including unligated nerves from each genotype) to better define the confidence interval for this estimate.

Task 1: Based on the results of these *in vivo* experiments, we realized that differences between Nf-/+ and WT tissues were going to be of a quantitative nature, and not easily quantified by either *in situ* hybridization or immunohistochemistry. So we decided to modify our initial approach and define differences between Nf-/+ and WT tissues, in the expression of members of the kinesin family of genes,

by qRT-PCR. We have selected the appropriate primers and have collected RNA from hippocampus and cortex of WT and Nf-/+ animals at 3-4 weeks age. We expect to complete these qRT-PCR studies in the ensuing months.

Task 2: We have also modified our approach to measuring axonal and dendritic transport in cultured neurons by live cell imaging. Because of technical difficulties with transfection of cortical or hippocampal neurons by electroporation (using the Amaxa nucleofector system), we have decided to develop a line of mice that are heterozygous knock-out at the Nf1 locus (i.e. Nf1-/+) and also express a transgene that expresses synaptophysin-YFP (Jackson Labs). This we hope to accomplish by mating the Nf-/+ animals with synaptophysin-YFP transgenic mice, and selecting for doubly transgenic mice. This line of mice will permit a number of experiments that will allow the direct monitoring of axonal and dendritic transport without transfection or immunostaining, and also permit testing of the effect of candidate drugs for their effect on axonal transport. We were not able to initiate this set of studies during the term of this pilot project, but expect that this will also be done during the coming year.

Key Research Accomplishments:

The main accomplishment of this research is obtaining support for our idea that there is a quantitative difference between Nf and WT animals in the rate of axonal transport. Based on our preliminary experiments, we suspect that there is at least a 20% decrease in the rate of transport in Nf-/+ axons.
Our preliminary results were presented as posters at the Children's Tumor Foundation meeting (June 2007) and at the Society for Neuroscience meeting (November 2007) (see abstracts attached in the appendix).

Reportable Outcomes:
We expect to submit our results for peer review and publication by late Summer 2008 – after completing further replicates of our experiments.

Conclusion:
There appears to be a quantitative decrease (20% or greater) in the rate of axonal transport in the peripheral nerves of Nf-/+ mice, as compared to wild-type litter mates.
Work to be done in the immediate future:
a) Additional replicates of the sciatic nerve ligation experiments.
b) qRT-PCR studies of kinesins in Nf and WT brain tissue.
Future studies planned:
a) In a follow-up to this study, we propose to test this hypothesis directly in the brain of wild-type and Nf-/+ mice, in vivo, using the method of Manganese Enhanced MRI (MEMRI) imaging to quantify axonal transport along the olfactory pathways, *in vivo*. We hope to eventually develop a safe and accurate method for measuring axonal transport in the human olfactory pathway, and directly determine if there is a correlation between cognitive function and rate of axonal transport in our patients with neurofibromatosis 1.
b) Develop the Nf1-/+ ; synaptophysin-YFP mouse model which will allow us to monitor axona and dendritic transport in live cells, and also serve as an experimental system in which we can test candidate drugs for their effect on axonal transport.

References:

1) Hakimi M-A, Speicher DW, Shiekhattar R. "The motor protein kinesin-1 links neurofibromin and merlin in a common cellular pathway of neurofibromatosis", J Biol Chem 2002, 277: 36909-36912.

2) De Schepper S, Boucneau IMA, Westbrook W, Mommaas M, Onderewater J, Messiaen L, Naeyaert J-MAD, Lambert JLW. "Neurofibromatosis type 1 protein and amyloid precursor protein interact in normal human melanocytes and colocalize with melanosomes", J Invest Derm, Advance online publication 5 Jan 2006; doi: 10.1038/sj.jid.5700087.

3) Donarum EA, Halperin RF, Stephan DA, Narayanan V. "Cognitive dysfunction in Nf1 knock-out mice may result from altered vesicular trafficking of APP/DRD3 complex", BMC Neuroscience 2006, 7: 22; doi:10.1186/1471-2202-7-22.

4) Yonekawa Y, Harada A, Okada Y, Funakoshi T, Kanai Y, Takei Y, Terada S, Noda T, Hirokawa N. "Defect in synaptic vesicle precursor transport and neuronal cell death in KIF1A motor protein-deficient mice", J. Cell Biol 1998, 141: 431-441.

Appendices/Bibliography of Abstracts-Publications:

Abstracts of posters presented at the Children's Tumour Foundation meeting (June 2007) and at the Society for Neuroscience meeting (November 2007). Attached below is the abstract submitted to the SFN meeting.

1) "Axonal Transport in the Nf1-/+ Knock-out Mouse". Shannon Snyder, Stephen Gabe Rice, Garilyn Jentarra, Johana Vallejo and Vinodh Narayanan, Barrow Neurological Institute.

2) "Axonal Transport in the Nf1-/+ Knock-out Mouse". Shannon Snyder, Stephen Gabe Rice, Nishit Srivastava, Garilyn Jentarra, Johana Vallejo, and Vinodh Narayanan, Barrow Neurological Institute.

Poster, Annual Meeting of the Society for Neuroscience, November 2007

"Axonal Transport in the Nf1-/+ Knock-out Mouse".
Shannon Snyder, Stephen Gabe Rice, Nishit Srivastava, Garilyn Jentarra, Johana Vallejo and Vinodh Narayanan
Barrow Neurological Institute, Phoenix, AZ

Mutation of the Nf1 gene (and loss of neurofibromin function) results in constitutive activation of the Ras signaling pathway. Heterozygous knock-out mouse models for NF1 (Nf-/+) display deficits in visuospatial learning and memory, also related to Ras pathway activation. Neurofibromin also functions to modulate other Ras-independent cellular signaling pathways (such as the adenylate cyclase-cAMP pathway) and loss of neurofibromin might affect many different cellular processes in neurons and glia. A number of studies have identified novel neurofibromin protein-protein interactions. These include association between neurofibromin and microtubules, neurofibromin and the kinesin-1 heavy chain (KHC/KIF5B) [1], and neurofibromin and amyloid precursor protein [2]. The heterozygous Nf1-/+ knock-out mouse exhibits decreased expression of several kinesin superfamily members [3]. APP itself is a receptor for kinesin light chains, and is important for the anterograde axonal transport of membranous vesicles.

These observations led us to hypothesize that the primary mutation in the Nf1 gene leads to defects in kinesin-mediated axonal and dendritic transport, and this may contribute to the defects in synaptic plasticity and the cognitive disturbance characteristic of NF1.

We have begun a series of experiments examining axonal transport in sciatic nerves of 2-4 week-old wild-type and heterozygous Nf-/+ mice. After anesthesia, both sciatic nerves are exposed, and a ligature

is placed around the sciatic nerve in the distal thigh. After 3-6 hours, the animals are euthanized, and sciatic nerves removed. Sciatic nerves are snap frozen and blocked for sectioning and immunohistochemistry using antibodies against tubulin, synaptophysin, syntaxin 1, and synaptotagmin. Intensity of staining with these synaptic markers in the segment immediately proximal to the ligature in wild-type and Nf-/+ nerves is compared. In separate experiments, after nerve ligation, a 3 mm segment of nerve immediately proximal to the ligature (D1 = distal 1), a 3 mm segment immediately distal to the ligature (D2 = distal 2), and a 3 mm segment at the origin of the nerve from the spine (P=proximal), are removed. Nerve segments from 6 wild-type and 6 Nf-/+ nerves were pooled for Western blot analysis with the antibodies against tubulin, syntaxin 1, synaptophysin and synaptotagmin. If anterograde axonal transport is unimpaired, then synaptic proteins would accumulate in the D1 fraction. The intensity of the specific bands (normalized to intensity of tubulin band) in the proximal and distal segments are compared, in Nf-/+ and wild-type nerves.

A copy of this poster is attached as page 7.

Personnel Supported by this Grant:
Shannon M. Snyder (Olfers), BS, MFS; Research Technician (100% effort on this project).

Supporting Data:

Attached below is a copy of the poster presented at the Society for Neuroscience meeting in November 2007 (with preliminary results of immunohistochemistry and Western blotting), as a portable image document).

Axonal Transport in the Nf 1 -/+ Knock-out mouse

Shannon L. Snyder, Stephen Gabe Rice, Nishit Srivastava, Garilyn Jentarra, Johana Vallejo, and Vinodh Narayanan

Barrow Neurological Institute, St Joseph's Hospital and Medical Center, Phoenix, AZ.

Abstract

Mutation of the Nf1 gene (and loss of neurofibromin function) results in constitutive activation of the Ras signaling pathway. Heterozygous knock-out mouse models for NF1 (Nf-/+) display deficits in visuospatial learning and memory, also related to Ras pathway activation. Neurofibromin also functions to modulate other Ras-independent cellular signaling pathways (such as the adenylate cyclase-cAMP pathway) and loss of neurofibromin might affect many different cellular processes in neurons and glia.

A number of studies have identified novel neurofibromin protein-protein interactions. These include association between neurofibromin and microtubules, neurofibromin and the kinesin-1 heavy chain (KHC/KIF5B) [2], and neurofibromin and amyloid precursor protein [3]. The heterozygous Nf1-/+ knock-out mouse exhibits decreased expression of several kinesin superfamily members [4]. APP itself is a receptor for kinesin light chains, and is important for the anterograde axonal transport of membranous vesicles.

These observations led us to hypothesize that the primary mutation in the Nf1 gene leads to defects in kinesin-mediated axonal and dendritic transport, and this may contribute to the defects in synaptic plasticity and the cognitive disturbance characteristic of NF1.

We have begun a series of experiments examining axonal transport in sciatic nerves of wild-type and heterozygous Nf-/+ mice. After anesthesia, both sciatic nerves are exposed, and a ligature is placed around the sciatic nerve in the distal thigh. After 5 hours, the animals are euthanized, and sciatic nerves removed. Sciatic nerves are snap frozen and blocked for sectioning and immunohistochemistry using antibodies against tubulin, synaptophysin, syntaxin 1, and synaptotagmin. Intensity of staining with these synaptic markers in the segment immediately proximal to the ligature in wild-type and Nf-/+ nerves is compared. In separate experiments, after nerve ligation, a 3 mm segment of nerve immediately proximal to the ligature (D = distal) and a 3 mm segment at the origin of the nerve from the spine (P=proximal), are removed. Nerve segments from wild-type and Nf-/+ nerves were pooled for Western blot analysis with the antibodies against tubulin, syntaxin 1, synaptophysin and synaptotagmin. The intensity of the specific bands (normalized to intensity of tubulin band) in the proximal and distal segment are compared, in Nf-/+ and wild-type nerves. This approach has been used to demonstrate impairment of axonal transport in KIF1A knock-out mice [5].

Supported by grants from DOD (CDMRP) and the Barrow Neurological Foundation

Neurofibromin and Axo-dendritic Transport

- Faxt axonal transport – vesicular proteins, mitochondria, membranous components, signaling proteins
- Neurofibromin colocalizes with microtubules, and with mitochondria
- Neurofibromin is found (in brain tissue) in cell bodies, axons, and dendrites
- Neurofibromin is part of a protein complex that includes kinesin heavy chain (KHC)
- Neurofibromin interacts directly with APP, a receptor for kinesin light chain (KLC), and is involved in anterograde transport of vesicles containing β-secretase and presenilin
- Neurofibromin enhances fast axonal transport of mitochondria, which is important for synaptic potentiation
- Ras signaling pathway is important for synaptic plasticity (AMPA-R trafficking)

Materials and Methods

Animals and genotyping: The Nf1 mouse colony was established at our institution with breeders originally obtained from Jackson Laboratory (*Nf1*^{tm1Fcr}) [1]. Mice were genotyped by PCR using primers (Neo.For GCGTGTTCGAATTCGCCAATG and Exon32.Rev GAAGGACAGCATCAGCATG . Control primers detected a segment of the MeCP2 gene, a Y-chromosome target (for sexing).

Animal surgery for sciatic nerve ligation: All experiments involving animals have been approved by the St. Joseph's Hospital and Medical Center IACUC. 3-4 week old mice were anesthetized by intraperitoneal injection of a ketamine / xylazine / acepromazine cocktail. Temperature was maintained using a low-heat electric heating pad, and anesthesia was maintained with periodic SQ injections of the anesthetic cocktail. The hind legs and back were shaved and sterilized with ethanol. The sciatic nerve was exposed from the spinal column to the popliteal fossa. A ligature was placed about 4 mm above the distal end of the dissected nerve, using a 6.0mm micro aneursym clip (Harvard Apparatus). Muscle and skin were reapposed, irrigated with sterile saline, and the skin incision covered with saline-saturated gauze to keep the area moist. After a period of 5 hours, the entire sciatic nerve (spinal column to distal end) was removed and snap frozen. Nerves were embedded for sectioning or segmented for Western blot analysis. Three separate 3 mm segments were harvested from each nerve: P = proximal (at the spinal column); D1 = 3 mm immediately proximal to the ligature; D2 = 3 mm immediately distal to the ligature. For wild-type animals, the nerve segments were pooled and snap frozen. For mice from Nf1 litters, the nerve segments were frozen separately, and pooled after genotyping result was known. Mice were euthanized at the end of the experiment and a piece of liver was removed for DNA extraction and genotyping.

Antibodies: The following antibodies were obtained – anti-tubulin III (Abcam), anti-syntaxin-1 (Sigma), anti-synaptophysin (Abcam), anti-synaptotagmin (Abcam). Secondary antibodies for Western blotting were conjugated to HRP (Novus). Alexa 488 conjugated anti-rabbit and Alexa 594 conjugated anti-mouse (Molecular Probes) secondary antibodies were used for immunohistochemistry.

Western blot analysis: Dilutions of primary and secondary antibodies that were best for Western blotting were determined first using mouse brain lysates. Sciatic nerve segments were homogenized in lysis buffer (0.1% SDS, 0.5%NP40, 0.5% deoxycholate, and 10% protease inhibitor cocktail in PBS) using a teflon homogenizer, and centrifuged at 4°C, 45,000 g for 10 minutes. Protein concentration in the supernatant was determined using BCA protein assay kit (Pierce). Proteins in each lysate were separated by SDS-PAGE and transferred to nitrocellulose membranes (BioTrace NT, Pall Co.). Blots were first stained with MemCode Reversible Protein Stain kit (Pierce) to assess equal loading and efficiency of Western transfer. Blots were then blocked with 1% BSA in PBS, incubated overnight at 4°C with primary antibody, washed and incubated for 1 hour at RT with secondary antibody conjugated to HRP, then developed with a SuperSignal West Pico Chemiluminescence kit (Pierce). Blots were then exposed to X-ray film, and images scanned.

Immunohistochemistry: Sciatic nerves were frozen and embedded in Tissue Tek cryomedium. 10 micron sections were cut on a cryostat, and kept at -20°C. Sections were fixed in 4% paraformaldehyde, washed in PBS, permeabilized, then blocked using Image IT (Molecular Probes). Primary antibodies were added and allowed to incubate overnight at 4°C, followed by washing and a 2 hour incubation in fluorescent labeled secondary. In control experiments, no primary antibody was used. Slides were coverslipped and imaged by laser confocal microscopy (Zeiss Pascal 5).

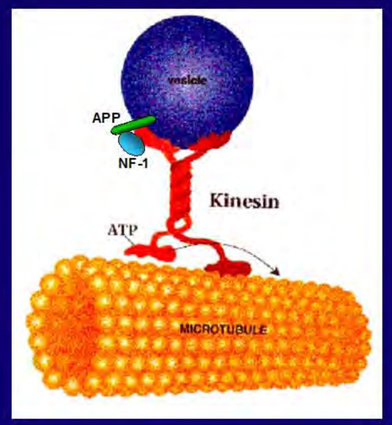


Figure modified from P.Pincus, U of California Santa Barbara

Results

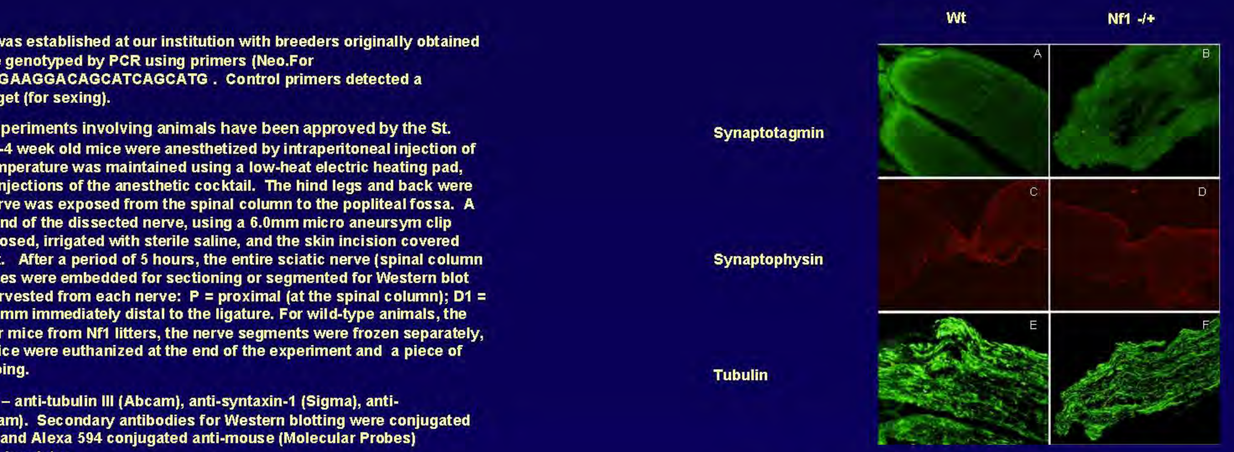


Figure 1. Immunohistochemistry of dissected sciatic nerves (Wild-type and Nf). Stained with antibodies against synaptotagmin (A and B), synaptophysin (C and D), and tubulin (E and F).

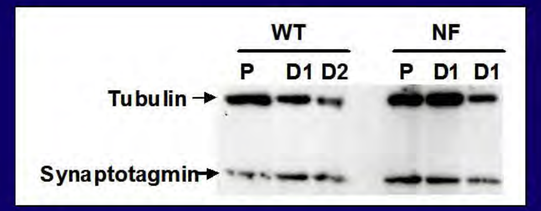


Figure 2. Western blot analysis of sciatic nerve segments: Each lane contains 25 ug of protein; blots were stained with antibodies against tubulin III and synaptotagmin. (P= Proximal – at spinal column; D1= immediately proximal to ligature; D2= immediately distal to ligature)

Conclusions

1. Immunohistochemical studies are suggestive of differences between Wt and Nf1 -/+ nerves in fast axonal transport of synaptic proteins .
2. We are able to measure tubulin, syntaxin, and synaptotagmin levels in pooled 3 mm segments of the mouse sciatic nerve.
3. Ratio of Synaptotagmin:Tubulin (S/T) at the D1 segment appears to be smaller in NF mice than in WT.
4. Differences between Wt and Nf1 -/+ levels of synaptic proteins in the distal segment (D1) are likely to be quantitative, and need to be repeated to assess statistical significance.
5. We plan to use anti-APP and mitochondrial markers to examine differences in APP and mitochondrial transport between Wt and Nf1 -/+ animals.

Selected References

1. Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA. Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. Nature Genetics. 1994, 7:353-61.
2. Hakimi M-A, Speicher DW, Shiekhattar R. The motor protein kinesin-1 links neurofibromin and merlin in a common cellular pathway of neurofibromatosis. J Biol Chem 2002, 277:36909-36912.
3. DeStefoppier S, Bouroneau IMA, Westbrook W, Mommaas M, Ondere vater J., Messiaen L, Naeyaert J-MAD, Lambert JLW. Neurofibromatosis type 1 protein and amyloid precursor protein interact in normal human melanocytes and colocalize with melanosomes. J Invest Derm 2006; doi:10.1038/sj.jid.5700687.
4. Donarum EA, Halperin RF, Stephan DA, Narayanan V. Cognitive dysfunction in NF1 knock-out mice may result from altered vesicular trafficking of APP/DRD3 complex. BMC Neuroscience 2006, doi:10.1186/1471-2202-7022.
5. Yonekawa Y, Harada A, Okada Y, Funakoshi T, Takei Y, Terada S, Noda T, Hirokawa S. Defect in synaptic vesicle precursor transport and neuronal cell death in KIF1A motor protein-deficient mice. J Cell Biol 1998, 141: 431-444.